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(54) HUMAN INTERFERON-BETA GENE.

(57) Human interferon-β gene of human chromosom origin, DNA containing said gene and DNA participating in control of transcription of said gene, and recombinant DNA between said DNA and vector DNA. Said gene and DNA can be introduced into cells of eukaryote to produce human interferon-β by the cells.

EP 0 070 906 A1

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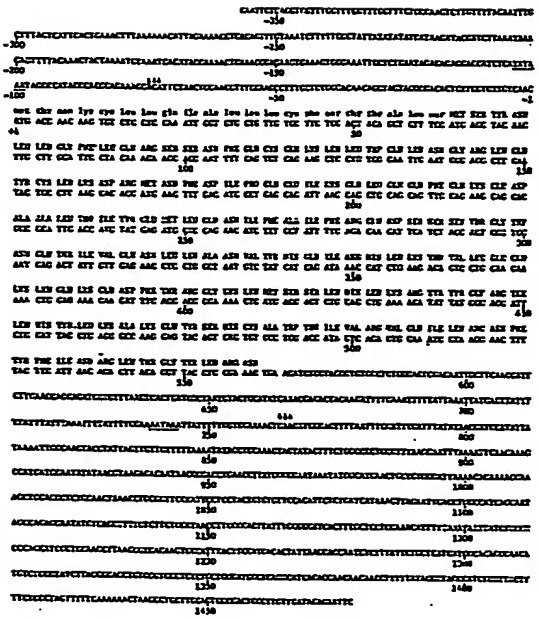

Specification

Title of the Invention

Human interferon-β gene

Technical Field

The present invention relates to human interferon-β gene derived from human chromosome [DNA (deoxyribonucleic acid) corresponding to entire region coding for interferon-β gene], a DNA containing said gene and a DNA responsible for control of transcription of said gene and a recombinant DNA of said DNA and a vector DNA.

Background Art

It has been known that a cDNA of human interferon-β is prepared using mRNA as a template. [Gene, 10, 11 - 15, (1980)]

Disclosure of the Invention

The present inventors have studied about the mass production of interferon using a recombinant DNA wherein human interferon gene is inserted into a plasmid DNA, for example, a plasmid DNA derived from Escherichia coli or a phage DNA, for example, λphage DNA derived from Escherichia coli by recombinant DNA technology.

As a result, it has been found that human interferon-β can be produced by propagating and multiplying a novel recombinant in bacteria such as Escherichia coli and a compound having the same structure as human interferon-β can also be produced by inserting the recombinant into a chromosomal gene of eukaryotic cells such as mouse cells or a virus and incorporating the resultant recombinant into eukaryotic cells. Thus the present invention has been

completed.

The recombinant DNA is a novel one having at least an entire region coding for human interferon-β gene in chromosome and furthermore containing a region which is believed to be responsible for the control of transcription.

In the present invention, human interferon-β gene and a DNA containing the same and a DNA responsible for control of its transcription are recovered directly from human chromosomal gene.

The present invention is explained in detail below.

The present invention relates to a human interferon-β gene derived from human chromosome, a DNA containing said gene and a DNA responsible for control of its transcription and a recombinant DNA of the DNA and a vector DNA.

The recombinant DNA of the present invention is prepared by the following procedure.

An entire DNA of human chromosome such as chromosomal DNA extracted from human fetal liver is cut with a restriction endonuclease in a proper length.

All fragments or selected fragments with proper length are concentrated by electrophoresis, etc. The fragments are inserted into a vector DNA by recombinant DNA technology to obtain recombinant DNAs. The novel recombinant DNA having human inteferon-β gene in chromosome is selected and isolated from the recombinant DNAs using as a probe the recombinant DNA containing a DNA showing complementarity to the human interferon-β messenger RNA, i.e. human inteferon-β cDNA, and being labelled with a radio isotope.

The process for producing said recombinant DNA is specifically explained more in detail.

Human chromosomal DNAs are extracted from human fetal liver with phenol, etc. The extracted DNAs are subjected to partial digestion with restriction endonucleases such as HaeIII, AluI, etc, to cut appropriately.

The thus obtained entire DNA fragments of human chromosome are joined with EcoRI linker, etc. and inserted into a DNA such as phageλ of Escherichia coli, using bacteriophage T_4 ligase to obtain recombinant DNAs.

The recombinant DNAs are modified to more infectious λphages by packaging method. The thus obtained whole recombinant DNAs containing entire human genes are called human gene library.

The human gene library contains almost all human gene DNAs as apparent from the construction method and most of the human genes can be isolated therefrom.

Since cleavage map for restriction endonucleases around human interferon-β gene in chromosome has been determined as mentioned below, the human whole gene library as a starting human interferon-β gene may be replaced with the following more concentrated recombinant.

That is, the entire human chromosomal DNA is digested completely with restriction endonuclease HindIII, etc. and subjected to agarose gel electrophoresis to obtain DNA fragments of about 10 kilobase (hereinafter referred to as Kb).

A DNA library having a length of about 10 Kb and HindIII cleavage sites at both ends is obtained by inserting the obtained fragments into λphage described above. Human interferon-β gene in chromosome is included in the DNA of about 10 Kb in the DNA library which seems to be concentrated about 10-fold over the whole gene library.

As the vector, Charon phages, plasmids such as pBR322, pCR1, pMB9, pSC1, and the like may be used instead of λphage.

A recombinant DNA having DNA fragment containing human interferon-β gene may be selected from the thus obtained human gene library as follows.

A recombinant plasmid having a structure (cDNA) which is complementary to human inteferon-β messenger RNA is isolated from Escherichia coli χ 1776 / TpIF319-13 ATCC 31712 according to the method of Currier and Nester [Analyt. Biochem. Vol. 76, 431 - 441 (1976)]. The plasmid

labelled with [^{32}P] according to nick translation method [Roop et al. Cell 15, 671 - 685 (1978)] is used as a probe.

The gene library prepared using Escherichia coli phage as a vector as mentioned above is dispersed on an agar plate and DNAs in phage plaques corresponding to each clone are fixed on a filter according to the method of Bentonn and Davis [Science, 196, 180 - 182 (1977)].

The filter is subjected to hybridization using the probe mentioned above and a cloned phage having a DNA hybridizing with the recombinant having a structure complementary to human interferon-β messenger RNA is isolated by radioautography.

The thus obtained phage is propagated and a DNA is extracted therefrom. The DNA is treated with restriction endonuclease such as EcoRI and the mixture is fractionated by agarose gel electrophoresis. The obtained fractions are fixed on a filter according to Southern method [J. Mol. Biol. 98, 503 - 517 (1975)]. Hybridization is carried out using the probe described above, for so called Southern blotting analysis (the same reference as mentioned above).

Thus, a phage clone having EcoRI fragment of 1.8 Kb which hybridizes with the cDNA is obtained.

More detailed restriction endonuclease map is prepared by the method of Smith and Birnstiel [Nucleic Acids Res. 3, 2387 - 2398 (1976)].

Further, DNA base sequence is determined according to, for example, the method of Maxam and Gilbert [Proc. Natl. Acad. Sci. USA 74, 560 - 564 (1977)]. The DNA base sequence is compared with that of human interferon cDNA [Gene 10, 11 - 15 (1980)] whereby the obtained clone is confirmed to have a chromosomal gene corresponding to human interferon-β messenger RNA, that is, human interferon-β gene in chromosome.

The human interferon-β gene and a DNA containing the gene and a DNA responsible for control of its transcription are recovered from the recombinant DNA obtained as above according to the method of Benton and Davis

[Science, 196, 180 - 182 (1977)] or the method of Grunstein-Hogness [Proc. Natl. Acad. Sci. USA 72, 3961 - 3965 (1975)].

Brief Description of the Drawings

Figure 1-a shows a restriction endonuclease map of the 15 Kb chromosomal DNA fragment cloned in λHIFN-β_1-121. Broken line in the figure indicates the arms of vector DNA derived from Charon 4A.

Figures 1-b and 1-d show a restriction endonuclease map of 1.8 Kb EcoRI fragment derived from human chromosomal DNA. Black box shows the region from which the messenger RNA is transcribed.

Figure 1-c shows a region in human chromosomal DNA corresponding to interferon-β cDNA. The open box in the figure indicates a protein coding region.

Figure 1-e shows strategy for sequence determination. Arrows in the figure indicate the direction and extent of the sequence of each fragment analysed.

The following restriction endonucleases in Figure 1 are described in the literature shown below.

Eco RI	:	Methods Mol. Biol. 7, 87 (1974)
Bgl II	:	Nucleic Acids Res., 3, 1747 (1976)
Hind III	:	J. Mol. Biol., 92, 331 (1975)
Bam HI	:	J. Mol. Biol., 97, 123 (1975)
Pst I	:	Nucleic Acids Res. 3, 343 (1976)
Pvu II	:	Gene 8, 329-343 (1980)
Hinf I	:	J. Mol. Biol., 110, 297 (1977)
Alu I	:	J. Mol. Biol., 102, 157 (1976)
Hae III	:	J. Virol., 10, 42 (1972)
Taq I	:	Proc. Natl. Acad. Sci. USA, 74, 542 (1977)
Ava II	:	Biochem. J., 159, 317 (1976)
Hin II	:	Gene 8, 329-343 (1980)
Eco RII	:	Nature New Biol., 244, 7 (1973)

Figure 2 shows the base sequence of 1.8 Kb Eco RI fragment. The section numbered +1 to +561 indicates the sequence coding for the human interferon-β protein, the arrows at -73 to -75 indicate the initiation site for transcription and the underline indicates the TATA box.

Best Mode for Carrying Out the Invention

The specific embodiment of the present invention is explained below.

Example 1

Human gene library prepared by the following method was provided by Tom Maniatis (California Institute of Technology).

An entire chromosome DNA is extracted from human fetal liver with phenol, etc. and subjected to partial digestion with restriction endonucleases HaeIII and AluI.

About 18 - 25 Kb fragments in the obtained DNA fragments are concentrated according to sucrose density gradient centrifugation method. Then, the fragments are connected to the arm DNA of Escherichia coli phageλ Charon 4A using a synthesized short chain nucleotide having a cleavage site for restriction endonuclease EcoRI to prepare an infectious phage recombinant DNA. Then, the recombinant is modified to a complete phageλ particle according to packaging method for the purpose of enhancing the infectiousness. The resultant human gene library is in principle believed to be an assembly of recombinants containing 18 - 25 Kb human DNAs containing almost all human genes.

The recombinant phage having a DNA fragment containing human interferon-β gene was selected from the human gene library using as a probe [^{32}P]-labelled cDNA fragment having the entire cDNA region translatable to human interferon-β protein by the method of Benton and Davis [Science 196, 180 - 182 (1977)]. Details are shown below.

A DNA fragment of about 0.57 Kb having the cDNA region translatable to human interferon-β protein and being used as a probe was prepared and radio-labelled as follows.

TpIF319-13 plasmid DNA is isolated from Escherichia coli χ 1776 / TpIF319-13 ATCC 31712 having the recombinant plasmid TpIF319-13 containing the human interferon-β cDNA by the method of Currier and Nester [Analyt. Biochem. 76, 431 - 441 (1976)] and digested with restriction endonucleases HincII, BglII and HhaI. The longest DNA fragment in the digest, i.e. 0.57 Kb DNA is the desired DNA fragment which is isolated by agarose electrophoresis according to the method of Tabak and Flavell [Nucleic Acids Research 5, 2321 - 2332 (1978)].

The DNA fragment is radio-labelled with [^{32}P] according to nick translation method [for example, Roop et al, Cell 15, 671 - 685 (1978)]. That is, 0.5 µg of the DNA was incubated at 15°C in 30 µl of aqueous solution containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 µM dGTP, 150 µM dTTP, 1 ng of DNase I (product of Worthington), [^{32}P]-α-dCTP (100 µCi, 2000 - 3000 Ci / mmol, product of RCC Amersham) and 15 units of DNA polymerase I (product of Boehringer Mannheim) for 4 hours. Then, EDTA is added to a final concentration of 20 mM and the mixture is incubated at 65°C for 10 minutes to inactivate the enzyme. After removal of proteins with phenol, the mixture is subjected to Sephadex G-50 (product of Pharmacia Fine Chemicals) column chromatography for desalting. The thus obtained [^{32}P]-radio-labelled cDNA fragment to be used as a probe has about 10^8 cpm/µg radioactivity.

Using as a probe the DNA-fragment prepared by radio-labelling the human interferon-β cDNA fragment, a recombinant phage having the DNA fragment containing human interferon gene is selected from the human gene library as follows.

The phage λ particles are dispersed on an agar plate [Science 202, 1279 - 1284 (1978)] to form phage plaques, the density of which is 10,000 to 30,000 per plate

with a diameter of 15 cm.

A nitrocellulose paper (available from Schleicher and Schull) is put on the agar plate and marked for the definition of direction. The agar plate is allowed to stand at 4°C for about 20 minutes to adsorb phages on the paper. The plate is kept at 4°C and the nitrocellulose paper is air-dried at room temperature for about 90 minutes. It is soaked in an aqueous soltuion consisting of 0.1N NaOH and 1.5 M NaCl for about 20 seconds to denature the phage DNAs. Then, they are neutralized in a solution of 0.2 M Tris-HCl (pH 7.4) and 2 x SSC (SSC means an aqueous solution consisting of 0.15 M NaCl and 0.015 M sodium citrate and 2 x SSC means 2-fold concentrated SSC) for about 20 seconds and additionally in 2 x SSC for about 20 seconds. After air-drying at room temperature for one hour and at 80°C for 3 hours, the denatured phage DNAs are fixed on the nitrocellulose paper.

Hybridization of the phage DNAs on the nitrocellulose paper prepared above is carried out using the radio-labelled human interferon-β cDNA as a probe as follows.

The nitrocellulose paper is incubated in 3 x SSC at 65°C for 30 minutes and in a 3 x SSC solution containing 0.2 % polyvinylpyrrolidone (product of Nakarai Kagaku Co.), 0.2 % bovine serum albumin (product of Iwai Kagaku Co.) and 0.2 % Ficoll (product of Pharmacia Fine Chemicals) at 65°C for 60 minutes. Then the paper is incubated at 65°C in a hybridization solution consisting of 1 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% SDS, and 100 µg/ml ultrasonicated and heat-denatured Escherichia coli DNA for 60 minutes, whereby whole hybridization treatment is completed.

Separately, the radio-labelled probe DNA is denatured by heating at 95°C for 10 minutes. Then, the pretreated nitrocellulose paper and the heat-denatured probe DNA are incubated in the hybridization solution mentioned above at 65°C for hybridization. After 12 to 18 hours, the nitrocellulose paper is picked up, washed with

2 x SSC twice, incubated at 65°C in a solution containing 0.3 x SSC and 0.1 % SDS for 60 minutes twice, air-dried at 80°C for one hour and subjected to radioautography using an X-ray film.

The radioautogram is put on the agar plate kept at 4°C and the phages hybridized with the probe are scraped up. The procedure is repeated and recombinant phages having the DNA hybridized with human interferon-β cDNA are purified to monoclones.

Thus, 11 clones are obtained by screening about 100 million phage plaques.

The recombinant DNA of each clone is prepared by the method of Maniatis [Cell, 15, 687 - 701 (1978)] and used in the following analysis.

The recombinant DNA of each clone is cleaved with restriction endonuclease EcoRI and chain lengths of DNA fragments formed are determined by agarose gel electrophoresis. The DNA digests of all clones have 20 Kb and 11 Kb DNA fragments derived from the arms of vector phage λ Charon 4A as well as some DNA fragments derived from human chromosomal DNA. By the analysis, 11 clones are classified to 5 species. Further, the Southern hybridization [Southern, J. Mol. Biol. 98, 503 - 517 (1975)] is carried out using human interferon-β cDNA probe employed in the screening mentioned above to determine which DNA fragments obtained by the digestion with EcoRI hybridize with human interferon cDNA.

That is, the DNA in each phage clone is digested with EcoRI and subjected to agarose gel electrophoresis. Thereafter, gels are recovered by cutting and incubated at room temperature in an aqueous solution containing 0.5 N NaOH and 1 M NaCl to denature the DNA. The same procedure is repeated in an aqueous solution containing 0.5 N Tris-HCl (pH 7.0) and 1.5 M NaCl to neutralize the gel. The gel is put on a filter paper soaked with 20 x SSC and a nitrocellulose paper is put on the gel. Then, a filter paper and a paper towel are put on the nitrocellulose paper to adsorb the denatured DNA in the gel thereon. After 12 to

18 hours, the nitrocellulose paper is peeled from the gel and air-dried at 80°C for 3 hours to fix the DNA on the nitrocellulose paper. Hybridization is carried out in the same manner as in the screening of the phage mentioned above.

Thus it is confirmed that four species of the human chromosomal gene fragments in the five species have a 1.8 Kb DNA fragment formed by the treatment with EcoRI, referred to as EcoRI fragment hereinafter, and the 1.8 Kb EcoRI fragment has a structure complementary to human interferon cDNA. The other species is confirmed to have a DNA fragment containing a part of the 1.8 Kb EcoRI fragment.

In the 11 clones, one of those which form 1.8 Kb EcoRI fragment is named λHIFN-β_1-121 and a cleavage map for restriction endonucleases is prepared based on the experiments using restriction endonucleases such as HindIII, BamHI, BglII, PstI and the like. The map is illustrated in Fig. 1-a.

In order to investigate the 1.8 Kb EcoRI fragment showing complementarity to the human interferon-β cDNA, the fragment is recloned using plasmid pBR322 as a vector as follows.

1 µg of λHIFN-β_1-121 DNA is digested with restriction endonuclease EcoRI and the cohesive ends with EcoRI are rendered flush with 5 units of DNA polymerase Klenow fragment (product of Boehringer Mannheim) in 30 µl of an aqueous solution containing 0.1 M potassium phosphate buffer solution (pH 6.9), 6 mM $MgCl_2$, 6 mM mercaptoethanol, 1 mM ATP and 1 mM TTP. After removal of proteins with phenol, the residue is treated at 37°C with 30 µl of a reaction solution consisting of 1 µg of DNA, 0.14 M potassium cacodilate (pH 7.6), 0.03 M Tris, 0.1 mM dithiothreitol, 1 mM $CaCl_2$, 1 mM dCTP and 2 units of terminal transferase for 15 minutes to tail the 3' ends of the EcoRI fragment with about 100 deoxycytidine residues. Separately, a vector is prepared by cutting pBR322 with PstI and tailing

the 3' ends of the PstI fragment with about 100 deoxyguanine residues. 0.05 µg of the thus obtained EcoRI fragment of human chromosomal gene DNA and 0.05 µg of pBR322 DNA are incubated at 65°C for 2 hours, at 45°C for one hour, at 37°C for one hour and at room temperature for one hour in a solution consisting of 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), and 5 mM EDTA to hybridize them. Escherichia coli χ 1776 is subjected to transformation with the reaction mixture by the method of Enea et al. [J. Mol. Biol. 96, 495 - 509 (1975)]. Tetracycline resistant strains are picked up and the DNA of 400 strains are fixed on a nitrocellulose paper [Grunstein-Hogness method, Proc. Natl. Acad. Sci. USA 72, 3961 - 3965 (1975)]. Hybridization is carried out on the nitrocellulose paper in the same manner as in the above screening of phages and Southern hybridization wherein a DNA is fragmented by heating in alkali in a hybridization solution and heat-denatured pBR322 DNA is added in a concentration of 30 µg/ml, using the same probe of human interferon-β cDNA to select the Escherichia coli strains having a recombinant plasmid containing 1.8 Kb EcoRI fragment.

A recombinant plasmid DNA having 1.8 Kb EcoRI fragment containing a recombinant DNA hybridizing with human interferon-β cDNA is prepared from the thus obtained Escherichia coli strain by the method of Currier and Nester mentioned above and analysed as follows.

It is apparent from the above that 1.8 Kb EcoRI fragment derived from the human chromosomal DNA contains a DNA complementary to the messenger RNA of human interferon-β DNA. For further clarification, a cleavage map for restriction endonucleases is prepared by measuring by agarose electrophoresis, etc. the chain lengths of the DNA fragments formed by cutting the recombinant plasmid DNA or a part thereof with one or more restriction endonucleases or by partially digesting the fragment labelled with [^{32}P] at the 3' ends using polynucleokinase [Smith and Birnstiel, Nucleic Acids Res., 3, 2387 - 2398 (1976)] (Fig. 1-b, -d).

Fig. 1-c shows the region corresponding to interferon-β cDNA wherein the open box indicates the protein coding region. The same region as the cDNA is found in the cleavage map. It is apparent from the foregoing that 1.8 Kb EcoRI DNA fragment derived from human chromosomal DNA has the same sequence as that of human interferon-β messenger RNA, i.e. cDNA and that 1.8 Kb EcoRI DNA fragment contains human interferon-β gene in chromosome (the black box in Fig. 1-b).

It has been revealed that intervening sequences, introns, present in the gene of many eucaryotic cells are absent in the human interferon-β gene. The absence of intervening sequences in the interferon-β gene in 1.8 Kb EcoRI fragment suggests that the gene DNA is applicable to the production of interferon proteins by procaryotic organisms not having the mechanism removing the intervening sequence such as Escherichia coli.

In order to prove the foregoing, the base sequence of the 1.8 Kb EcoRI fragment is determined by the method of Maxam and Gilbert [Proc. Natl. Acad. Sci. USA 74, 560 - 564 (1977)]. The result is shown in Fig. 2. The 1.8 Kb EcoRI fragment introduced into Escherichia coli has been deposited with the American Type Culture Collection in U.S.A. as Escherichia coli CI4 under accession number ATCC 31905.

Claims

(1) Human interferon-β gene derived from human chromosome.

(2) A DNA containing human interferon-β gene derived from human chromosome and a DNA responsible for control of its transcription.

(3) A recombinant DNA of a DNA containing human interferon-β gene derived from human chromosome and a DNA responsible for control of its transcription, and a vector DNA.

(4) The recombinant DNA according to claim 3, wherein the vector DNA is selected from λphage, Charon phages, and plasmid pBR322, pCR1, pMB9 and pSC1 derived from Escherichia coli.

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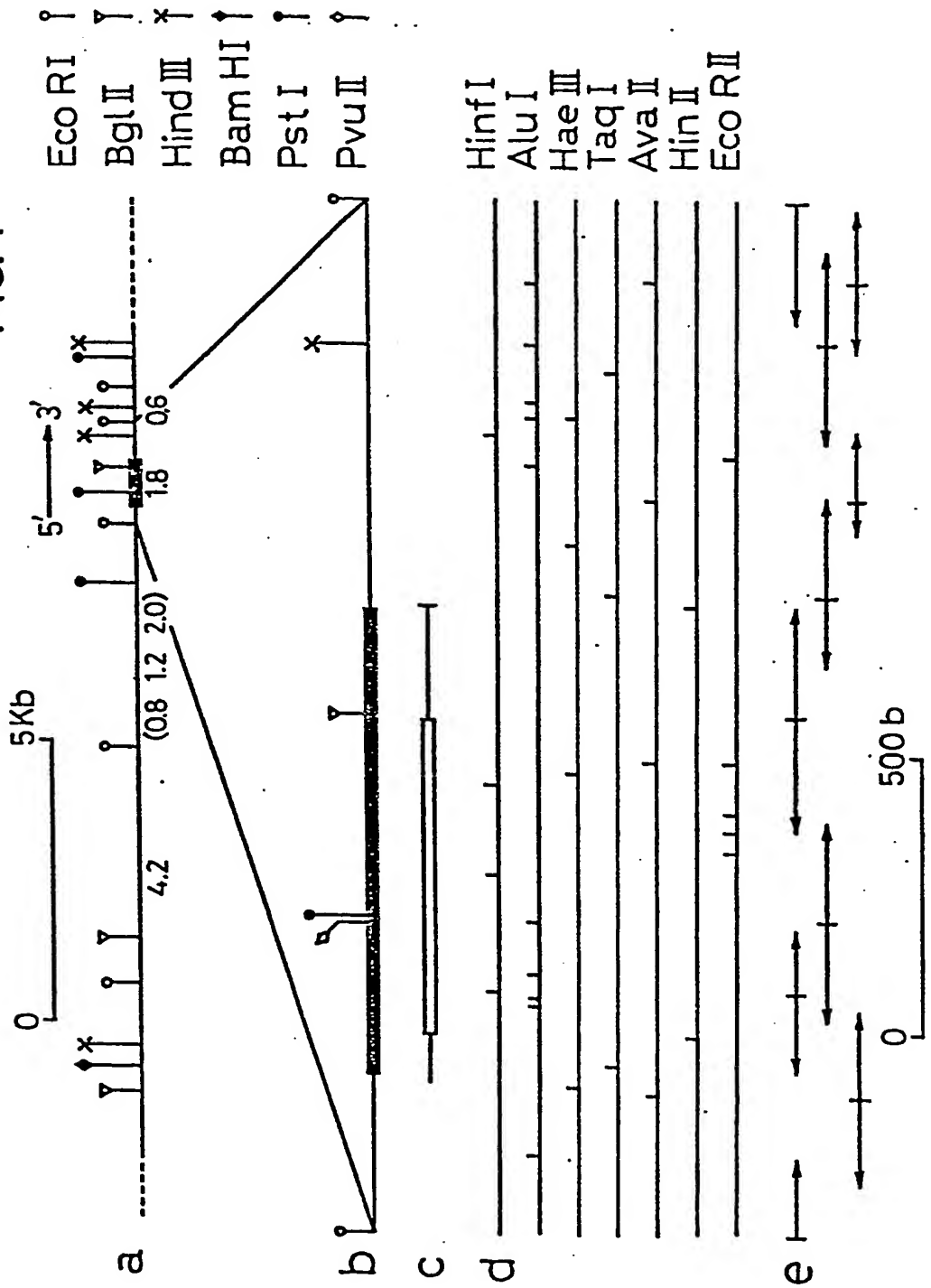
FIG. 1



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INTERNATIONAL SEARCH REPORT

International Application No PCT/JP82/00094 0070806

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] C07H21/04, C12N15/00// C12P19/34, 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System 5	Classification Symbols
I P C	C07H21/04, C12N15/00, C12P19/34, 21/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 6

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A	Nature, Vol. 285, No. 19 (June, 1980) P542 - 549. Especially see 547-549	1 - 4
E	JP,A, 57-24400 (G.D. Searle and Co.) 8. February. 1982 (08.02.82), Column 38, lines 6 to 20, column 55, line 17 to column 57, line 11	1 - 4
P	Ishikawa Kunihiko Henshu "(Bessatsu Tanpakushitsu Kakusan Koso) Interferon Kenkyu no Shinpo" 1. December. 1981 (01.12.81) Kyoritsu Shuppan Kabushiki Kaisha P169 - 182, Especially see page 174 to 175	1 - 4

* Special categories of cited documents: 15

"A" document defining the general state of the art

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
April 28, 1982 (28.04.82)	May 10, 1982 (10.05.82)
International Searching Authority 1	**Signature of Authorized Officer 20**
Japanese Patent Office	